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(57) Abstract

Processes for the production of isoflavones are described wherein plant material from plants of the genus *leguminosae* are contacted with water, a C_1–C_{10} organic solvent, and optionally an enzyme which cleaves isoflavone glycosides to the aglucone form, so as to form a combination, incubating the combination for a time sufficient to allow isoflavones of the aglucone form to partition into the organic solvent, and thereafter recovering isoflavones from the organic solvent.

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PREPARATION OF ISOFLAVONES FROM LEGUMES

Isoflavones are plant chemicals which occur largely in members of the *Leguminosae* plant family. They are based on a simple diphenolic ring structure as described for example by Carlson et al (1980) Journal of Chromotography, 198, 193-197 (incorporated herein by reference).

Over 700 different isoflavones are described and these display a range of biological functions both within the plant and within animals including humans which eat the isoflavone-containing plants.

A small sub-group of isoflavones (daidzein, genistein, biochanin, formononetin and glycitein) are distinguished by their ability to bind to estrogen receptors on animal (including human) cells. This is due to the close similarity of the steric structure of the diphenolic rings f isoflavones with the steroidal ring structure of estrogens such as estradiol, estrone and estriol. Although having substantially lower binding affinity to the receptor compared to steroidal estrogens, estrogenic isoflavones are weakly estrogenic. This group also exhibits a range of biological functions in animal cells which appear to be independent of the estrogen receptor and these include anti-oxidant, diuretic, anti-spasmolytic and anti-cancer effects. These interesting functions with their potential therapeutic benefits has brought this particular group of isoflavones to the attention of medical researchers in recent years.

In the plant, the isoflavones can occur in a variety of forms - (i) in the basic aglucone form, (ii) as a glucone, being bound to a sugar moiety such as glucose via a β-glucosidic linkage (the glycosidec form), (iii) the glucone form + a malonyl moiety, and (iv) the glucone form + an acetyl moiety as described for example, by Carlson et al (1980) as referred to above.

The glycosidic form (either alone or in the malonyl or acetyl forms) is water-soluble and is the predominant form for the isoflavones in many legumes to facilitate transport and storage. Th glycosidic form provides enhanced stability to degradative factors such as heat, oxidation and ultraviolet irradiation. At the intra-cellular site of biochemical function of the isoflavone,

an intra-cellular β-glycosidase enzyme cleaves the sugar moiety where present, leaving the more biologically active, but substantially water-insoluble, aglucone form.

Isoflavones are fairly widely distributed within the plant kingdom although they are found predominantly in members of the *Leguminosae* family. The estrogenic isoflavones (genistein, biochanin, formononetin, daidzein, glycitein) follow this general rule in being largely restricted to the genus *Leguminosae*. Most legumes investigated have been found to contain at least detectable levels of one or more of these five estrogenic isoflavones but the richest sources are the legumes - soya, lentils, chick peas, fenugreek, clovers, alfalfa and various varieties of beans. The richest sources of these compounds are the clovers (including *Trifolium pratense*, *Trifolium sub-terranean*) and soya (either whole soya or defatted soya or any materials ensuing as products of soya processing including soya grits, soya hypocotyls and soy molasses). The levels of these compounds in clovers and soya varies according to the variety or cultivar and on seasonal, environmental and plant age factors. Levels in clovers vary between about 0.5 and 3.5% (on dry weight basis) and in soybeans between about 0.05 and 0.3% (dry weight).

Isoflavones may be used as therapeutics for pre-menstrual syndrome and menopausal syndrome (US Patents 5569459, 5516528, 5498631) and osteoporosis (US Patent 5424331) and as food additives (US Patents 4366082, 4390559). Given these important uses, it is advantageous to isolate or to concentrate isoflavones from plants.

Various techniques have been proposed to achieve isolation of isoflavones, but essentially there are two distinct methods. The first method involves the conversion of the water-soluble glucone form to the water-insoluble aglucone form to facilitate the subsequent extraction of the aglucones in a suitable organic solvent such as alcohol. This conversion step is described as being achieved in one of two ways: either (a) through hydrolysis by exposure to vigorous heating (typically 80-100°C) at low pH (*Wang K, Kuan SS, Francis OJ, Ware KM, Carman AS. "A simplified HPLC method for the determination of phytoestrogens in soybean and its processed products." J. Agric. Food Chem. 38:185-190, 1990*); or (b) by exposure to an enzyme (glucose hydrolase, β-glycosidase or β-glucur nidase) which specifically cleaves the

β-glycosidic linkage with the sugar moiety. The enzyme either is added to the reaction or the naturally occurring β-glucosidase within the plant can be utilised. In respect to the latter, a method is described (JP 89-345164/47) whereby the natural enzyme activity within soybeans is utilised by heating soyflour to 45-55°C for several hours, although the amount of naturally-occurring enzyme activity in commercially available soyflour samples is highly variable and even when at its maximum is insufficient to obtain hydrolysis of more than about 50-60% of the glucones present.

The various hydrolysis reaction procedure (either enzymatic or heating/low pH) are described as being applied to an admixture of ground plant material in water. At the conclusion of the hydrolysis process, the aqueous phase is separated from the undissolved plant material to facilitate the next step. Once the conversion of the glucone to the aglucone form is achieved, the aqueous mixture then is contacted with an organic (and water immiscible) solvent. The aglucones due to their substantial water insolubility are extracted into the organic solvent phase and subsequently recovered.

Previously proposed methods involve initial water extraction of the isoflavones in their glycosidic form so that they either are retained in this form or can be converted subsequently to their aglucone form. Techniques described for this approach involve adding the ground plant material to water and over a period of time (several hours to several days) the naturally-occurring glycosidic forms of the isoflavones dissolve in the aqueous phase. After separating the undissolved plant material from the aqueous phase, the isoflavones in the aqueous phase are converted to the aglucone form by any of the methods outlined above and subsequently recovered. A variant of this approach involves selective removal of the aglucone forms from the aqueous mixture by absorption on to an appropriate ion-exchange resin. The isoflavones subsequently are eluted from that resin using a water:organic solvent mixture, concentrated by rotary evaporation, and then hydrolysed to the aglucone form by enzymatic digestion or heat/acid treatment (JP 95-272884/36).

Disadvantages f the above techniques include (a) a multiplicity of steps, (b) the use of vigorous treatments such as heating and/or strong acid and/or strong alkali, (c) the

comparatively low yields of isoflavones, (d) the very high cost of hydrolysing enzymes, and (e) the high capital costs and high running costs associated with large-scale multiple step extraction of isoflavones in commercial quantities. All of the current known isoflavone extraction procedures are affected by one or more of these disadvantages and serve to greatly reduce the commercial viability of the process. If the purported community health benefits of the estrogenic isoflavones are to be realised then they must become economically accessible to the general community. For this to happen, an improved and more cost-effective method of extraction must be found.

Summary of the Invention

In the broadest aspect of this invention there is provided processes for the production of isoflavones from plants of the genus *Leguminosae* which comprises contacting plant material with water, a C_1-C_{10} organic solvent and optionally an enzyme which cleaves isoflavone glycosides to the aglucone form, to form a combination, and incubating the combination for a time sufficient to allow isoflavones of the aglucone form to partition into the organic solvent, and thereafter recovering isoflavones from the organic solvent.

The combination of the aforementioned components may comprise an aqueous phase containing enzyme and plant material and an organic phase into which the isoflavones partition. The combination may alternatively comprise an emulsion formed by vigorous mixing of the organic solvent and water, or if a water miscible organic solvent is used the combination is a mixture of water and organic solvent.

Where the organic solvent is non-water miscible the organic solvent containing dissolved isoflavones may be removed, for example by evaporation to give an isoflavone containing residue. The residue may then be mixed with a C_1-C_{10} organic solvent in which isoflavones are substantially insoluble such that isoflavones precipitate and are subsequently recovered.

Where the organic solvent is miscible with water, the organic solvent in the combination may be removed, for example by evaporation, to give an isoflavone containing residue and water

which may be thereafter mixed with a non-water miscible C_1-C_{10} isoflavone solubilising organic solvent to give an organic and an aqueous phase. The organic solvent phase containing dissolved isoflavones may be collected and isoflavones recovered therefrom. The organic solvent may be evaporated with water addition whereafter isoflavones form a water insoluble flocculate which is subsequently recovered.

Where an enzyme is used to cleave isoflavone glycosides to the aglucone form it preferably includes a β-glucanase. More preferably the enzyme is a mix (or combination) of β-glucanase and β-xylanase.

In another aspect there is provided a composition comprising isoflavones when produced according to the process of this invention.

Detailed Description of the Invention

The present invention provides in its broadest aspect a process for the production of isoflavones from plants of the genus *Leguminosae* which comprises contacting plant material with water, a C_1-C10 organic solvent and optionally an enzyme which cleaves isoflavone glycosides to the aglucone form, to form a combination and incubating the combination for a time sufficient to allow isoflavones of the aglucone form to partition into the organic solvent, and thereafter recovering isoflavones from the organic solvent.

The combination which results from combining together the plant material, water, a C_1-C_{10} organic solvent and optionally an enzyme which cleaves isoflavone glycosides to the aglucone form, may be in the form of a separated phase mixture comprising an aqueous phase containing the enzyme and plant material and an organic phase into which isoflavones partition on incubation following cleavage by the enzyme. The combination may comprise an emulsion formed by vigorous mixing of the organic solvent and water, or where the organic solvent is water miscible the combination may be a mixture of water and organic solvent. Where the combination comprises an emulsion it is preferred to remove particulate material from the emulsion, after a period of time sufficient to enable the aglucone form of

the isoflavone to partition into the organic solvent, using a standard separation process such as filtration or centrifugation. Phase separation then occurs, this subsequently allowing recovery of isoflavones from the organic solvent component.

Where the organic solvent is non-water miscible the organic solvent component containing dissolved isoflavones may be removed, for example by evaporation to give an isoflavone containing residue. The residue may then be mixed with a C_1-C_{10} organic solvent in which isoflavones are substantially insoluble such that isoflavones precipitate and are subsequently recovered.

Where the organic solvent is miscible with water, the organic solvent in the combination may be removed, for example by evaporation, to give an isoflavone containing residue and water which may be thereafter mixed with a non-water miscible C_1-C_{10} isoflavone solubilising organic solvent to give an organic and an aqueous phase. The organic solvent phase containing dissolved isoflavones may be collected and isoflavones recovered therefrom. The organic solvent may be evaporated with water addition whereafter isoflavones form a water insoluble flocculate which is subsequently recovered.

The enzyme optionally used to cleave the isoflavone glycoside to the aglucone form (hereinafter referred to as isoflavone) is required specifically to cleave the β-glycosidic linkage which is described as the dominant linkage between the isoflavone and its carbohydrate (normally glucose) moiety. A person skilled in the field of carbohydrate chemistry would deduce that the most appropriate enzyme to achieve this would be a β-glucosidase and possibly a β-glucanase. As Table 1 shows, in an experiment to compare the relative potencies of different carbohydrate enzymes in their ability to cleave the glycosidic linkage of soy isoflavones, it was found that β-glucosidase was highly effective; β-glucuronidase was found unexpectedly also to be highly effective; β-glucanase unexpectedly was found to have relatively low potency and required a considerably longer reaction time. In some isoflavone containing plants such as clovers, endogenous β-glycosidase enzyme activity is generally sufficient to effect cleavage of the glucone form without the need for

additional cleavage enzymes. Hence, enzyme addition may, in the process of this invention, be regarded as opti nal.

Table 1.

Comparative actions of different carbohydrate-acting enzymes in converting soya isoflavones in their glycosidic forms (daidzin, genistin) to the aglucone forms (daidzein, genistein).

Enzyme type*	Relative activity (% conversion)
β-glucosidase	90
β-glucuronidase	98
β-glucanase	40
1,4-bD-glucan hydrolase	0
1,4-a-D-glucan hydrolase	0
β-xylanase:b-glucanase (10:1)	85
β-xylanase:b-glucanase (1:1)	87

* All enzymes added at the same concentration to a standard amount of isoflavone.

A β-glucanase/β-xylanase enzyme mix was found by the inventors to be relatively effective in cleaving the isoflavone glycoside to the aglucone form. This was entirely unexpected given that there was no reason to expect that a β-xylanase would have any effect on the described form of glycosidic linkage on the isoflavone glucone form. Advantageously, this fungal-derived enzyme mix is available in large commercial quantities at an advantageous cost. Although only slightly less efficient than the more specific b-glucosidase and β-glucuronidase enzymes, the latter enzymes are not available in bulk, commercial quantities or at cost-effective prices. Moreover, the low cost of the commercial β-glucanase/β-xylanase enzyme mix allowed the dosage per unit of isoflavone to be increased which more than compensates for the slightly lowered efficiency. In one embodiment of the process involving an enzyme, the organic solvent does not cause significant inactivation of the enzyme used.

The plant material is derived from plants f the genus *Leguminosae* and may be obtained from plants such as soy, clover (including subterranean clover, red clover, and other isoflavone-containing clovers), chickpeas, lentils, beans (such as broad, haricot, kidney, lima and navy beans) which generally contain higher levels of isoflavones than other plants of the genus *Leguminosae*. It is preferred that the plant material be derived from soy or clover although this is not to say that other isoflavone containing plants of the genus *Leguminosae* may not be used in the process of this invention. Where isoflavones are extracted from clovers, the use of an enzyme which cleaves isoflavone glycosides is unnecessary.

The plant material is preferably in fine particulate form, such as a flour produced by grinding or otherwise processing plant material such as clover, soy beans, other beans, chickpeas and lentils. The preferred plant material is soya (*Glycine max*) or clover, such as red clover. Without limiting the present invention, it is preferable to remove as much as possible of parts of the plant that do not contain isoflavones to any great extent in order to reduce the bulk of material to be exposed to the extraction process. For example, about 90% of the isoflavones contained in harvested clovers occurs in the leaves and about 10% in the stalks and petioles so it is advantageous to separate the leaves from the stalks which can be achieved by first exposing the dried plant to a threshing action followed by differential sieving to separate the smaller leaves from the larger stalks. In another example, soybeans may be dehulled and/or defatted and dehulled. Defatted soyflour is readily available in commercial quantities. In another example, soy hypocotyl which often breaks away from the soy cotyledons during regular dehulling processes and is readily collected by standard methods such as sieving, contains typically higher isoflavone levels (between about 0.5 and 1.5%) compared to the whole soybean (between about 0.05 and 0.3%).

The organic solvents utilised in the various embodiments of this invention comprise from 1 to 10 carbons (C_1-C_{10}) and include water-immiscible and water-miscible organic solvents. Water-miscible organic solvents include C_1-C_{10} alcohols such as methanol, ethanol, propanol and isopropanol, acetic acid, acetone, acetonitrile, dimethyl formamide, dimethyl sulphoxide, n-propanol, isopropanol, tetrahydrofuran and mixtures of any such solvents. Water-immiscible C_1-C_{10} solvents which are isoflavone solubilising include C_4-C_{10} alcohols (such as

butanol, hexanol and pentanol), C_1-C_{10} alkoxy solvents (such as ethyl methyl ketone, methyl phenyl ketone, hexane-2,4-dione and the like); C_2-C_{10} esterified acids (such as ethyl acetate, ethyl methyl malonate, dimethyl phosphonate); C_1-C_8 aldehydes (including butanone, pentanone, hexanedial, cyclohexane carbaldehyde and butane-1,2,4-tricarbaldehyde); C_2-C_{10} ethers, C_2-C_3 alkenes, C_2-C_4 alkanes or phenol and its derivatives (such as benzene 1,2,4-thiol) and mixtures of any such solvents. Organic solvents in which isoflavones are substantially insoluble include C_5-C_{10} alkanes (such as hexane, cyclohexane, heptane and octane) and C_4-C_{10} alkenes and mixtures of any such solvents. The organic solvent utilised is preferably selected to have a volatility to enable the organic solvent to be removed by evaporation (for example by distillation, rotary evaporation and the like) so that isoflavone compounds dissolved in the organic solvent can be subsequently recovered.

The water used in the process may be from any conventional water source, distilled water, deionized and distilled water or the like. The water may contain preservatives to retard microbial growth and/or other additives as are well known in the art. The respective proportions of water and organic solvent are not limiting on this invention. Generally equal proportions of water and organic solvent are used, although the ratio of water to organic solvent may vary, for example from 1:10 to 10:1.

Where the combination resulting from the mixture of the water and organic solvent comprises an organic phase and an aqueous phase the respective phases may be gently mixed or agitated. This can easily be achieved by a vertically disposed stirrer which allows mixing of the respective phases without intermixing of the phases as such.

The process of the invention does not require elevated temperatures and may be conducted under ambient temperature conditions, for example from 5°C to 35°C. Ambient temperature conditions can therefore suffice without the need for sophisticated temperature control as is required in prior art processes where elevated extraction temperatures are necessary.

In one embodiment the extraction process of the present invention is a one pot, single stage reaction which confers substantial benefits such as cost savings in capital equipment expenditure and in time. The efficiency of enzymatic digestion and solvent extraction in one step, according to one embodiment of this invention, is very efficient and gives high yield of isoflavone products, which is generally in contrast to prior art procedures.

Isoflavone compounds are recovered from the organic solvent component generally by vaporisation (evaporation) of the organic phase such as by rotary evaporation, distillation or the like. A small amount of oil containing the aglucone isoflavones is found to remain following removal of the organic phase. This isoflavone-enriched oil may be regarded as the desired end-product and used as such, although it is preferable to continue the extraction process to further concentrate the isoflavones. The oil containing isoflavones may be then removed by the addition of a suitable organic solvent such as hexane, heptane and octane which are highly soluble for oils but very low solubility for isoflavones; hexane preferably is used because of its relatively low cost. The solvent (such as hexane) is added at a ratio to the oil of between about 1:1 and 50:1, preferably 10:1. It is found that the oil readily partitions into the organic solvent phase and that this is associated with the isoflavones falling out of solution and settling to the bottom of the vessel. The hexane:oil phase then is removed leaving the isoflavone-containing residue. This may be recovered and dried, such as in an oven at a temperature between about 50°C to 120°C, to produce a fine powder which is subsequently formulated for therapeutic use as described hereafter. Preferably, however, the hexane extraction step is repeated a further 1-3 times to effect complete removal of oil. Alternatively, the isoflavone containing oil may be subject to HPLC fractionation, ion exchange, chromatography or other techniques well known in the art for isoflavone enrichment/purification.

Where the C_1-C_{10} organic solvent used to extract plant material is miscible with water (for example an alcohol such as ethanol), the organic solvent may be removed by evaporation (such as rotary evaporation or distillation) to give a concentrate containing an isoflavone containing oil in water. This concentrate may be mixed with a C_1-C_{10} isoflav ne solubilising organic solvent, for example ethyl acetate to give an organic isoflavone containing phase and

an aqueous phase. The organic phase may be collected and isoflavones recovered therefrom. For example, organic solvent may be evaporated with water addition, for example using a still, whereafter isoflavones form a water insoluble flocculate which is subsequently recovered and formulated into a pharmaceutical/health composition.

At this stage the extracted material is of high isoflavone content, such as from 36 to 70% isoflavones, and generally is comparable to the ratio of isoflavones of the starting material. As a consequence the yields are typically very high. The material may be used for therapeutic purposes at that point, for example being dried and subsequently formulated, or can be subject to further processing as is known in the art to further purify the isoflavone. Further purification may comprise HPLC fractionation, ion exchange chromatography or other techniques as are well known in the art. For example, by PLC fractionation daidzein or genistein may be removed.

Where soya is the starting material, the isoflavones extracted are daidzein, genistein, and glycitein; the remaining material is composed of a range of compounds including phytosterols and other water-insoluble compounds. Where clover is the starting material, the isoflavones extracted are daidzein, genistein, formononetin and biochanin; various flavonoids including chlorophyll as well as phytosterols make up the bulk of the remainder of the isolate.

The isoflavones produced according to the process of this invention may be individually purified. For example daidzein and genistein may be purified to HPLC, or other chromatographic techniques or standard methods for purifying these compounds known in the art.

The isoflavones may be formed into pharmaceutical compositions or health compositions, drinks, foods and the like, in combination with appropriate excipients, carriers and the like as are well known in the art, for example as described in *Handbook of Pharmaceutical Excipients*, Second Edition, American Pharmaceutical Association, 1994 (incorporated herein by reference). Pharmaceutical compositions or health compositions may comprise tablets, capsules, powders for reconstitution, syrups and the like. Standard carriers/excipients used

in such formulations include microcrystalline cellulose, calcium hydrogen phosphate, magnesium stearate and colloidal silica. Foods containing isoflavones may comprise food bars, biscuits, snack foods and other standard food forms well known in the art. Drinks may contain flavouring, buffers and the like.

In another aspect there is provided a composition containing isoflavones when prepared according to the process of this invention, optionally in association with a pharmaceutically acceptable carrier and/or excipient. The composition may be in association with food components, for example in food or muesli bars, biscuits, drinks, and the like.

It would appear that the prior art has not contemplated the use of a one pot process for converting isoflavones from the glucone to the aglucone form at the same time as recovery of the aglucone isoflavones in an organic solvent for a number of reasons. It may have been believed necessary to remove residual leguminous plant material from the process after cleavage of the glycoside form. It may also have been regarded that organic solvent would inactivate enzymes which effect formation of the aglucone form. As a consequence, in the prior art conversion of the water soluble glucone form to the water insoluble aglucone form has been carried out in multiple steps, followed by a subsequent step of extraction of the aglucones in a suitable organic solvent.

Embodiments of the present invention will now be described with reference to the following non-limiting examples.

Example 1

2000 kg of defatted soyflour is placed in a 10,000 L vessel as depicted in Figure 1 containing 5,000 L of deionised water and 10 kg of β-glucanase/b-xylanase (Bio-Feed Beta CT; Novo Nordisk, Denmark). 1000 L of ethyl acetate is then layered on top of the aqueous suspension to give a two phase combination. Both aqueous and solvent phases are gently mixed by continuous stirring using a vertical propeller mixer (Figure 1). It is found that at the point f contact between the aqueous and organic solvent phases, the aglucone isoflav nes readily

move from the aqueous to the organic solvent phase. The constant agitation of the aque us phase is designed to ensure maximum exposure of the hydrolysed isoflavones to the ethyl acetate; the constant agitation of the ethyl acetate helps to ensure a high isoflavone concentration gradient between the two phases, thereby maximising the rate of dissolution of the water-insoluble aglucone form into the ethyl acetate. An optional further contact between the two phases may be provided by circulating the lower aqueous suspension through the ethyl acetate phase.

After about 4 to about 48 hours, but preferably around 18 hours, the agitation and recirculation processes are stopped and the two phases allowed to separate maximally. The ethyl acetate is removed and evaporated using a still. About 20 L of oil remains unevaporated. 200 L of hexane is added to the oil and mixed vigorously by stirring for about 5 minutes. This is allowed to stand overnight (about 18 hours) without stirring and it is found that particulate material containing the aglucone isoflavones settles to the bottom of the reaction vessel. The hexane:oil phase is decanted leaving a sludge. A further 5 l of hexane is added to the sludge to effect removal of residual oil. This mixture is allowed to stand for 1 hour by which time the particulate material has settled out once again. The hexane:oil phase is decanted leaving a semi-solid sludge which is collected and dried in an oven at a temperature of about 85°C. By HPLC analysis this material is found to contain between about 36-70% (typically about 60%) isoflavones. Importantly, the ratio of the isoflavones in the extract is comparable to that of the starting material and the isoflavone yields typically are very high (Table 2). This material can be used for the purpose as is, or can be subjected to further processing in order to further purify the isoflavones.

Table 2

Recovery of isoflavones from whole soyflour using the extraction method described in Example 1.

Isoflavone	% recovery of starting material
daidzein	80.3
genistein	76.3
glycitein	75.0

Example 2

The starting material is 200 kg of soy grits containing a mixture of soy hypocotyls and pieces of soy cotyledons and representing a more enriched source of isoflavones (about 10.% compared to about 0.2% on whole soyaflour). 200 kg of soy grits is placed in a 3000 L vessel containing 1000 L of deionised water and 2.5 kg of glucan hydrolase (Bio-Feed Beta CT; Novo Nordisk, Denmark). 1000 L of ethyl acetate is then added and the aqueous and solvent phases then mixed together vigorously using a pump with a capacity of about 200 L per minute to ensure effective contact between the two phases, that is, form an emulsion. The mixing continues at room temperature for a period of between 1-24 hours, but preferably 4 hours. The particulate material in this combination is then separated from the liquid phase by a standard process such as filtration or centrifugation. The removal of the particulate material destroys the emulsion, and on allowing the resulting liquid phase to stand for about 30 minutes there is effected separation between the aqueous and the ethyl acetate phases. The ethyl acetate which contains the isoflavones then is removed and exposed to distillation. The residual oil remaining after distillation of the ethyl acetate then is treated according to the steps outlined in Example 1 above to isolate the isoflavone-enriched material.

Example 3

500 kg of clover is fed into a counter-current extraction unit and mixed with 5000 L of 50% ethanol for a period of 6 hours. The solvent extract is then pumped to storage and the clover discarded. The ethanol is then recovered by rotary evaporation under pressure (-80kPa) and at 80°C resulting in 500 L of extract concentrate (an isoflavone containing oil in water) and recovery f 4000 L of an ethanol/water mixture. The concentrate is mixed with ethyl acetate

at a ratio of 1:4 (i.e. 2000 L ethyl acetate) and the mixture left to settle into a water layer and an ethyl acetate layer. The isoflavones are solubilised into the ethyl acetate layer. The ethyl acetate layer is pumped into a still, and the solvent evaporated under vacuum with water addition. The wet floc (active component) is then pumped to a storage tank. 50% of the wet floc is then mixed with a spray drying agent, spray dried and active isoflavones recovered (25%). The remaining 50% is washed with hexane, dewatered, dried at 90°C, milled and mixed with carriers/excipients for tableting.

Example 4

The dried end product of Examples 1 to 3 above (Sample 1) can be used as starting material to concentrate genistein or daidzein with/without glycitein. Purification is established by standard procedures including HPLC, ion exchange chromatography and other chromatographic separation. In one series of experiments, 3 kg of the dried end product of Examples 1 to 3 is fractionated allowing separation of daidzein and genistein. Daidzein, of purity between about 95-99% (typically 98.5% purity) is isolated. Genistein of similar purity is recovered.

Example 5

Pharmaceutical compositions can be prepared from the products extracted according to the examples above.

1. The following composition is prepared in the form of a tablet:
   Using soyflour extract prepared according to Example 1 and containing (genistein 35% and daidzein 28% by weight)
   60 mg of extract
   340 mg of a standard tablet inert carrier

   This composition is tableted to provide a 400 mg tablet containing 20 mg of genistein and 17 mg of daidzein.

2. The following composition is prepared in the form of a capsule:
Using soy hypocotyl extract prepared according to Example 2 and containing (genistein 18%, daidzein 35% and glycitein 18% by weight)
60 mg of extract
190 mg of a standard pharmaceutical inert carrier

All contained in a non-toxic gelatin capsule and providing 200 mg containing approximately 11 mg of genistein, 21 mg of daidzein and 11 mg of glycitein.

3. The following composition is prepared in the form of a tablet:
Using a genistein extract prepared according to Example 4 and containing (genistein 99.5% by weight)
50 mg of extract
150 mg of a standard tablet inert carrier

This composition is tableted to provide a 200 mg tablet containing 50 mg of genistein.

4. The following composition is prepared in the form of a tablet:

A 500 mg tablet containing 40 mg of isoflavones prepared according to Example 3 and 460 mg inert excipients/carriers.

The carriers referred to above include cellulose (microcrystalline), calcium hydrogen phosphate, soy polysacchardie, magnesium stearate and silica-colloidal (anhydrous).

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer r step or group of integers or steps but not the exclusi n of any ther integer or step or group of integers or steps.

CLAIMS

1. A process for the production of isoflavones from plants of the genus *leguminosae* which comprises contacting plant material with water, a C_1-C_{10} organic solvent and optionally an enzyme which cleaves isoflavone glycosides to the aglucone form, to form a combination, incubating the combination for a time sufficient to allow isoflavones of the aglucone form to partition into the organic solvent, and thereafter recovering isoflavones from the organic solvent.

2. A process according to claim 1 wherein the organic solvent is recovered, the organic solvent removed to give an isoflavone residue, and the residue is mixed with an organic solvent in which isoflavones are substantially insoluble such that isoflavones precipitate and are subsequently recovered.

3. A process according to claim 1 wherein the organic solvent in the combination containing isoflavones dissolved therein is water-miscible and is removed to give an isoflavone containing residue and water, which is thereafter mixed with a non-water miscible C_1-C_{10} isoflavone solubilising organic solvent to give an organic and an aqueous phase, the organic solvent phase containing dissolved isoflavones collected and isoflavones recovered therefrom.

4. A process according to claim 3 wherein the organic solvent phase is evaporated with water addition whereafter isoflavones form a water soluble flocculate.

5. A process according to claim 1 wherein the combination comprises an aqueous phase containing an enzyme and plant material and an organic phase into which the isoflavones partition.

6. A process according to claim 1 wherein the combination comprises an emulsion formed by vigor us mixing of the organic solvent and water.

7. A process according to claim 5 wherein the enzyme is a β-glucanase and β-xylanase mixture.

8. A process according to claim 1 wherein the plant material is mixed with the water and an enzyme whereafter the organic solvent is added so as to form an organic phase and an aqueous phase, or an emulsion formed by vigorous mixing of the organic solvent and water.

9. A process according to claim 1 wherein the plant material is mixed with water, whereafter an enzyme is added with the organic solvent.

10. A process according to claim 1 wherein the plant material is from soy or clover.

11. A process according to claim 1 which is carried out at from 10°C to 30°C.

12. A process according to claim 1 wherein the plant material is in particulate form.

13. A process according to claim 12 wherein the plant material is soy flour.

14. A process according to claim 12 wherein the plant material is a variable mixture of soy hypocotyls and soy cotyledons.

15. A process according to claim 1 wherein the plant material is clover.

16. A composition comprising isoflavones produced according to claim 1 optionally in association with a pharmaceutically acceptable carrier and/or excipient.

17. A food composition comprising isoflavones produced according to claim 1 in association with food components.

18. A process according to claim 1 wherein daidzein is purified from the recovered isoflavones.

19. Isoflavones when prepared according to the process of claim 1.

20. A process according to claim 1 wherein genistein is purified from the recovered isoflavones.

21. Daidzein when prepared according to the process of claim 18.

22. Genistein when prepared according to the process of claim 20.

AMENDED CLAIMS

[received by the International Bureau on 28 August 1998 (28.08.98); original claim 1 amended; remaining claims unchanged (1 page)]

1. A process for the production of isoflavones from plants of the genus *leguminosae* which comprises contacting plant material with water, a C_1-C_{10} organic solvent and an enzyme which cleaves isoflavone glycosides to the aglucone form, to form a combination, incubating the combination for a time sufficient to allow isoflavones of the aglucone form to partition into the organic solvent, and thereafter recovering isoflavones from the organic solvent.

2. A process according to claim 1 wherein the organic solvent is recovered, the organic solvent removed to give an isoflavone residue, and the residue is mixed with an organic solvent in which isoflavones are substantially insoluble such that isoflavones precipitate and are subsequently recovered.

3. A process according to claim 1 wherein the organic solvent in the combination containing isoflavones dissolved therein is water-miscible and is removed to give an isoflavone containing residue and water, which is thereafter mixed with a non-water miscible C_1-C_{10} isoflavone solubilising organic solvent to give an organic and an aqueous phase, the organic solvent phase containing dissolved isoflavones collected and isoflavones recovered therefrom.

4. A process according to claim 3 wherein the organic solvent phase is evaporated with water addition whereafter isoflavones form a water soluble flocculate.

5. A process according to claim 1 wherein the combination comprises an aqueous phase containing an enzyme and plant material and an organic phase into which the isoflavones partition.

6. A process according to claim 1 wherein the combination comprises an emulsion formed by vigorous mixing of the organic solvent and water.

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00305

A. CLASSIFICATI N OF SUBJECT MATTER

Int Cl[6]: C07D 311/36 311/40 C12P 17/06 A61K 31/35

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC : C07D 311/36,311/40,C12P 17/06,A61K 31/35

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU : IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DERWENT WPAT: CA (STN) Keywords: DAIDZEIN or DAIDZIN or GENISTEIN or GENISTIN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Derwent Abstract Accession No. 95-272884/36, Class B02, JP 07173148 A (KIKKOMAN CORP) 11 July 1995 whole document	1, 6, 10, 12-14, 16, 19-20, 22
A	AU 78399/94 A (PROTEIN TECHNOLOGIES) 20 April 1995	1-22
A	AU 78002/94 (680554) B (PROTEIN TECHNOLOGIES) 20 April 1995	1-22

☐ Further documents are listed in the continuation of Box C

☒ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 June 1998	-2 JUL 1998
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer CEDRIC SCHAFFER Telephone No.: (02) 6283 2277

INTERNATIONAL SEARCH REPORT
Information on patent family members

International Application No.
PCT/AU 98/00305

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search rep rt. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
AU	78399/94	BR	9407820	CA	2174120	CN	1135222
		EP	794960	JP	9506076	US	5637562
		WO	9510529				
AU	78002/94	BR	9407822	CA	2173999	CN	1135214
		EP	723536	JP	9506077	US	5637561
		WO	9510512				

END OF ANNEX